Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio Technology 68 (2014) 22280-22283

ISSN: 2229-712X

Effect of salinity on the growth of petroleum hydrocarbons degrading *Bacillus sp.* isolated from chronically polluted ship breaking yards

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ARTICLE INFO

Article history:
Received: 19 January 2014;
Received in revised form:
22 February 2014;
Accepted: 5 March 2014;

Keywords
Salinity
Petroleum hydrocarbon
Biodegradation

ABSTRACT

The aim of this paper is to determine the effect of salinity on the growth of some previously known petroleum biodegrading *Bacillus sp*. under laboratory condition where petroleum hydrocarbons are the sole source of carbon. Bushnell-Hass (BH) mineral salt media consisting of different NaCl concentrations (0.0 to 0.4 mole/liter or ML^{-1}) were prepared and supplemented with 2% kerosene/diesel/engine oil. The media were then inoculated with the bacteria namely *Bacillus pasteurii, B. badius, B. cirroflagellosus, B. circulans and B. brevis* individually. After 7 days of incubation, bacterial growth was determined by measuring the optical density (OD) of the media at 620 nm. We found that salinity has a great impact on the growth of the bacteria under investigation. A NaCl concentration ranging from 0.05 to 0.3 ML^{-1} was found to have a positive impact on the growth of all 5 *Bacillus sp.* NaCl concentrations below and above the said range were found to be growth limiting. Interestingly our findings indicate that the maximum growth of a bacterium depends not only on the optimum salinity level but also on the type of petroleum hydrocarbons provided. The findings of this study are important for understanding the impact of salinity on the biodegradation process of petroleum hydrocarbons and for developing optimized application approaches to sweep such pollutants from contaminated sites.

Introduction

The unexampled population increase and industrial development of the modern era resulted in the increase of mainstream solid and liquid waste pollutants to decisive levels as well as created a variety of previously unknown pollution [1]. Contamination of the environment with crude oil above critical land results in pollution and warfare like gulf war (1991) released approximately 0.8 to 2 million metric tons of oil in the Persian Gulf [2]. The more recent BP oil spill, the largest accidental marine oil spill in the history, has caused extensive damage to marine and wildlife habitats as well as the Gulf's fishing and tourism industries [3].

Contamination of soil and groundwater with oil hydrocarbons is a global concern, especially in country as densely populated as Bangladesh because of their toxicity and recalcitrant properties. Ship breaking yards offer itself as the major source of petroleum hydrocarbon pollution and are rapidly growing especially in the coastal area of Chittagong, the port city of Bangladesh [4-6].

Biodegradation of oil in contaminated soil is one of the most feasible and economical methods for the treatment of oil contamination in environment [7-8]. Biodegradation of petroleum was also studied in extreme environments such as high salinity [9-11]. Many experiments about salinity and effects of high NaCl concentration on hydrocarbon biodegradation in liquid mediums have been reported [12-13]. Salt content is supposed to be an important factor in the bioremediation process of organic pollutants and it affects the bioremediation process by various ways including direct inhibition of metabolic activity because of unfavorable high osmotic potential of the microbe's environment, and altered solubility or sorption of toxic or essential ions [14].

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The inhibition of microbial growth increases with higher salt content, while the degradation of the substrate is much less inhibited by NaCl during dichloromethane degradation [15]. Several studies implied that inhibition of organic decomposition was generally found with increased salt content in different biological process of petroleum decomposition [16-18].

In this study, the effect salinity on the growth of five different petroleum biodegrading *Bacillus sp*. was investigated. In particular, the potentials of these bacteria to grow in media, where kerosene, diesel and engine oil were the only sole source of carbon, with different NaCl concentrations were evaluated.

Materials and methods

Petroleum hydrocarbons: Kerosene, diesel and engine oil, used in this experiment, had been collected from the ship breaking yards, Chittagong, Bangladesh.

Samples: Twenty different petroleum hydrocarbon contaminated composite samples (soil, water and combinations of soil and water) were aseptically collected from the ship breaking yards. The Samples were transported to the laboratory on an ice pack in a cooler box and stored at 4°C.

Culture Media: Nutrient agar medium (HiMedia Laboratories) was used for the routine culture of the isolates. Bushnell-Hass mineral salt media [19] possessing 0.02% MgSO4, 0.002% $CaCl_2$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.1% NH_4NO_3, 1 drop of concentrated $FeCl_3$ supplemented with 2% of respective petroleum hydrocarbons and with varying amount of NaCl concentrations (0.05, 0.1, 0.2, 0.3 and 0.4 ML^{-1}) were used for the salinity test. This medium was also termed as mineral salt medium in this writing.

Isolation, identification, selection and characterization of potential hydrocarbon degrading bacteria: Total bacterial load of the samples, identification and characterization of potential hydrocarbon degrading isolates and the degradation

potentials of those bacterial isolates were described previously [20]. Finally, ten bacterial isolates were confirmed as potential petroleum degraders. Among them five were found to belong to the genus *Bacillus sp.* namely *Bacillus pasteurii*, *Bacillus badius*, *Bacillus cirroflagellosus*, *Bacillus circulans* and *Bacillus brevis* and used in this study.

Effect of salinity on the growth of petroleum biodegrading *Bacillus sp.*: Bacterial inoculum was prepared by incubating few drops of bacterial suspension in nutrient broth in an orbital incubator (SI50, Stuart Scientific, UK), shaking continuously at 150 rpm (rotation per minute) for 18 hours at 37°C. The test begins by adding 2% (v/v) of the five different inoculum to the Bushnell-Hass mineral salt media individually and incubating at 37°C and 150 rpm. On 7th day the cultures were withdrawn and vortexed. The growths of different isolates in different NaCl concentrations were measured by determining the absorbance at 620 nm under visible light in a spectrophotometer (UV-VIS RS spectrophotometer, LaboMed. Inc.). Controls were maintained for each different salt concentration and petroleum hydrocarbon without inoculating the media with the bacterial isolates. The difference between treatment and control was taken as the real absorbance. The experiment was carried out in triplicate and mean values were expressed.

Results and Discussions

The effect of salt on the growth of *Bacillus pasteurii* varied with the type of petroleum hydrocarbon supplemented. When the hydrocarbons were kerosene and engine oil, this bacterium grew best where the NaCl concentration was 0.2 ML^{-1} and it was 0.1 ML^{-1} NaCl for diesel. A relatively better growth of this bacterium was observed when there was no salt in the medium than 0.4 ML^{-1} NaCl conc. The results showed that 0.5 to 0.3 ML^{-1} salt concentration had a positive effect on the growth of this bacterium in comparison to no salt at all in the media (Figure 1).

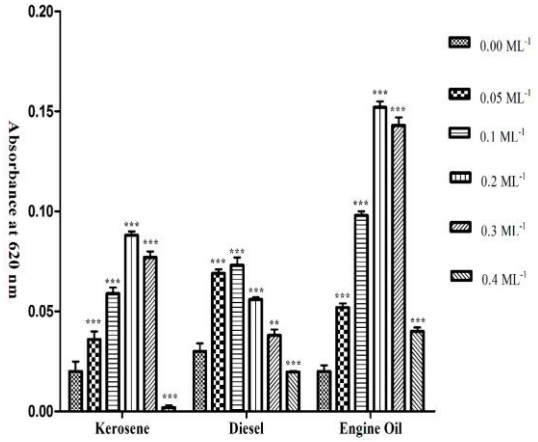

Figure 1: Effect of salt concentration on *Bacillus pasteurii* while grown in BH mineral salt medium supplimented with kerosene, diesel and engine oil respectively. The data is representative of three independent experiments. Here, * = P <0.05, ** = P< 0.01 and *** = P< 0.001, P = significance level.

The maximum growth of *Bacillus badius* depended both on the type of petroleum hydrocarbon and salt concentrations. This bacterium showed highest growth at 0.05, 0.1and 0.2 ML^{-1} NaCl concentrations in kerosene, diesel and engine oil supplemented media respectively. In kerosene supplemented medium, the growth increased three times at 0.05 ML^{-1} NaCl than media with no salt, but growth started to decrease sharply from 0.2 ML^{-1} NaCl onwards. In diesel-supplemented medium, beyond and above 0.1 ML^{-1} NaCl concentration, there was no convinced effect of salt on growth. On the other hand, in engine oil supplemented medium, 0.1, 0.2 and 0.3 ML^{-1} NaCl concentrations had almost the same irrefutable impact on the growth of *Bacillus badius* (Figure 2).

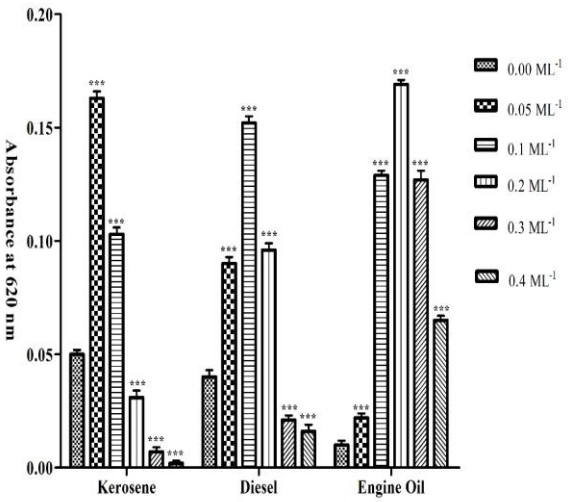

Figure 2: Effect of salt concentration on *Bacillus badius* while grown in BH mineral salt medium supplimented with kerosene, diesel and engine oil respectively. The data is representative of three independent experiments. Here, * = P <0.05, ** = P< 0.01 and *** = P< 0.001, P = significance level.

For *Bacillus cirroflagellosus*, 0.2 ML^{-1} NaCl had showed the maximum positive effect on its growth irrespective of the petroleum hydrocarbon supplemented. In kerosene medium, there was a five times decrease in growth when NaCl rose from 0.2 to 0.3 ML^{-1}. The growth was ten times higher in engine oil supplemented medium with 0.2 ML^{-1} NaCl than the same medium with no NaCl (Figure 3).

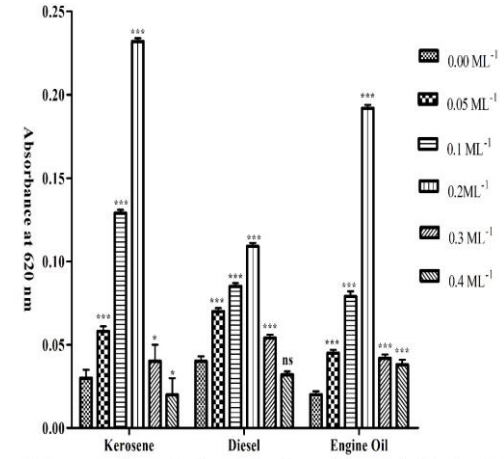

Figure 3: Effect of salt concentration on *Bacillus cirroflagellosus* while grown in BH mineral salt medium supplimented with kerosene, diesel and engine oil respectively. The data is representative of three independent experiments. Here, * = P <0.05, ** = P< 0.01 and *** = P< 0.001, P = significance level, ns = not significant.

The growth of *Bacillus circulans* had been doubled when NaCl conc. was increased from 0.0 to 0.2 ML^{-1} in diesel modified Bushnell-Hass medium. However, the growth of the bacterium reached its maximum at 0.05 ML^{-1} NaCl in kerosene and engine oil supplemented media. After that, the growth began to decrease with increase of salt concentrations. This bacterium can grow better in various salt concentrations and the optimum range is between 0.05 to 0.3 ML^{-1} (Figure 4).

From Figure 5, it can be said that higher salt concentration had a negative effect on the growth of *Bacillus brevis.* In kerosene-supplemented medium, the growth of this bacterium decreased ten times at 0.4 ML^{-1} NaCl in comparison to the same medium with no salt. When the hydrocarbons were diesel and engine oil, 0.1 and 0.05 ML^{-1}NaCl conc. respectively provided the maximum growth condition for this bacterium (Figure 5).

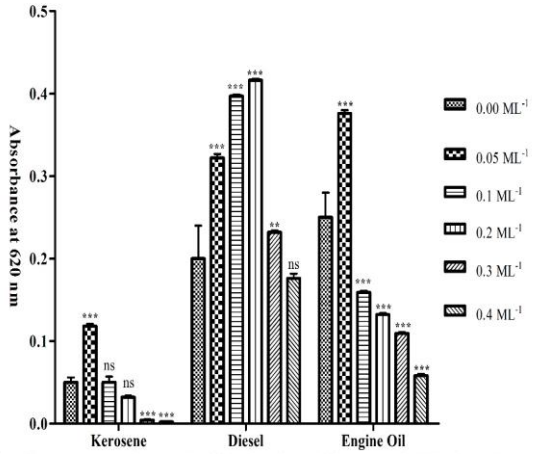

Figure 4: Effect of salt concentration on *Bacillus circulans* while grown in BH mineral salt medium supplimented with kerosene, diesel and engine oil respectively. The data is representative of three independent experiments. Here, * = P <0.05, ** = P< 0.01 and *** = P< 0.001, P = significance level, ns = not significant.

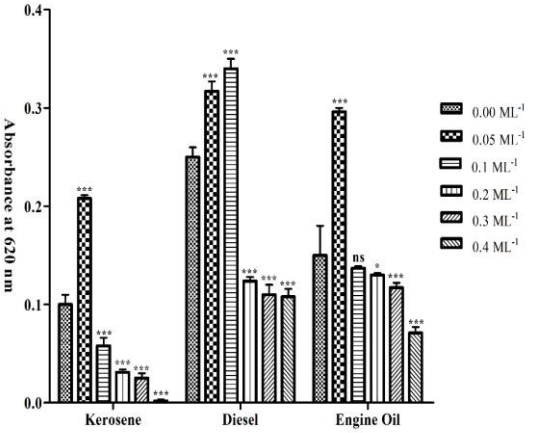

Figure 5: Effect of salt concentration on *Bacillus brevis* while grown in BH mineral salt medium supplimented with kerosene, diesel and engine oil respectively. The data is representative of three independent experiments. Here, * = P <0.05, ** = P< 0.01 and *** = P< 0.001, P = significance level, ns = not significant.

This current study, evaluating the effects of salinity on the growth of petroleum biodegrading *Bacillus sp.* had leaded us to some remarkable observations. We found that NaCl concentrations of 0.05 to 0.2 ML^{-1} had a positive effect on the growth of the bacteria with a little variation. The results of Mille *et al*. [16] were found in concurrence with our observation while investigating the biodegradation of Ashtart crude oil by a mixed bacterial community isolated from a marine sediment in varying concentration of NaCl (0 to 2 ML^{-1}). They reported that the amount of oil degradation increased initially with increasing salt concentrations to a maximum level for 0.4 ML^{-1} NaCl and thereafter the amount of oil degradation decreased with increasing salt concentrations. Our study also showed that salinity level like 0.4 ML^{-1} NaCl, which is equivalent to seawater, exerts a negative effect on the growth all the bacteria under study. We found salt concentrations higher than the optimum range influence the biodegradation of the petroleum hydrocarbons negatively and ultimately results in the lowest growth the bacteria. We can see this is very much similar with the findings of an experiment where it was showed that salinity had great impact on bioremediation and petroleum hydrocarbons in the saline-alkaline soil [21]. Researchers like Kargi *et al.* [22] and Woolard *et al.* [23] drew a conclusion that is very much similar to our findings. They found that biodegradation of oil by microorganisms in the presence of high NaCl and salinity was slow because high NaCl medium may disrupt cell membrane, denature some proteins such as enzymes, or change the osmotic force, which could be lethal for microorganisms and according to our findings, this typical high salinity level starts from 0.4 ML^{-1} NaCl.

Conclusion

This study clearly revealed that salinity had a great impact on the growth of some petroleum biodegrading *Bacillus sp*. The growth of those bacteria had been positively influenced within a certain range of NaCl concentrations. Beyond that range, salinity had a negative or no impact on the growth. From our study, it can be said that salinity has both positive and negative effect on the growth of petroleum biodegrading *Bacillus sp*. and interestingly the maximum growth depends not only on the optimum salinity level but also on the type of petroleum hydrocarbons provided. The findings of this study is important for understanding the impact of salinity on the biodegradation process of petroleum hydrocarbons and it will contribute to develop optimized application approaches to sweep such pollutants from contaminated sites.

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